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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Compound Screening Based on a Window of Chemical-Messenger-Independent Activity

(72) Dennis, Michael - Canada ;
Labrecque, Jean - Canada ;
Bouvier, Michel - Canada ;
Chidiac, Peter - Canada ;

(71) Same as inventor

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ABSTRACT

A method of testing chemical compounds for their abilities to inhibit chemical-messenger-independent activity of G protein-coupled receptors involving; expressing DNA encoding a G protein-coupled receptor in a cell expression system in such a manner as to generate a reproducible "window of chemical-messenger-independent activity" that allows for discrimination of chemical compounds based on relative ability to inhibit chemical-messenger-independent activity of said G protein-coupled receptor; measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-independent activity of said receptor in said system comprising whole cells or membrane fragments containing G protein, an appropriate effector, and cloned G protein-linked receptor; contacting a test-compound with the system under conditions permitting interaction of the test-compound with said receptor; and measuring the change, if any, of the quantifiable parameter which reflects the ability of the test compound to inhibit the chemical-messenger-independent activity of the G protein-coupled receptor.

COMPOUND SCREENING BASED ON A WINDOW OF CHEMICAL-MESSENGER-INDEPENDENT ACTIVITY

This invention relates to methods for screening compounds for their ability to interact with and modulate the functional properties of receptors coupled to guanyl nucleotide-binding regulatory proteins (G proteins). More specifically, it describes a method for testing and ranking compounds for their abilities to inhibit the chemical-messenger-independent activity of G protein-coupled receptors. This method is based upon the production of a "window of agonist-independent activity" (a.k.a. a "window of spontaneous activity" or "window of agonist-independent activity") by expressing cloned cDNAs encoding a G protein-coupled receptor of interest in cultured cells to generate a specific range of receptor concentration: concentrations lower than the window will not allow for ample signal detection to rank compounds on a statistically significant basis, whereas concentrations greater than this window are toxic to the cultured cell and generate variable results. Only receptor concentrations within the window allow for reliable, reproducible assay results that rank compounds on the basis of their abilities to exert inhibitory activity.

The following information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the following information constitutes prior art against the present invention. Moreover, publications referred to in the following discussion are hereby incorporated by reference in their entireties in this application.

One manner in which cells communicate with one another (either within an organism or between the environment and the organism) or receive information from the environment is by signalling via proteinaceous macromolecules, known as receptors, which are oftentimes imbedded in the outer surface of cell membranes. A chemical substance (either released from one cell within the organism or entering the organism from the environment) will interact with the receptor located on the cell (or within the cell) of the organism by binding to a specific area on the receptor, thereby producing a conformational change in the structure of the receptor. Depending upon where the receptor is located in a cell, and what other molecules and macromolecules it is in contact with, a specific biochemical change will be generated in the immediate vicinity by the change in receptor conformation, which can effect greater biochemical changes in the cell through a cascade or domino effect of subsequent chemical signalling. Examples of chemical messengers that are produced to act on receptors in this way to effect cell-cell communication are transmitters such as neurotransmitters, hormones and pheromones.

There are many different types of receptors, which fall within families of related proteins depending on features including their localization in the cell, their basic structural organization and the mechanism by which they transmit signals upon binding a chemical messenger. Three well-characterized classes of cell-surface receptor proteins are channel-linked, catalytic and G-protein-linked receptors. Channel-linked receptors form channels in a lipid membrane that will undergo a conformational change upon chemical-messenger binding, to open or close, either allowing specific ions to pass, or preventing them from passing through the membrane. The change in the flow of ions through the membrane can initiate significant changes in the environment of a cell such as the ion fluxes that

und rli signal transmission within a nerve cell. Catalytic r ceptors function as enzymes wh n activated by a chemical-mess ng r. Usually, th chemical-messeng r binds to a portion of the receptor that is exposed on the external surface of the membrane causing a portion of the enzyme to catalyze a reaction on the inner surface of the cell. G protein-linked receptors are proteins which function as one part of a multi-component complex whereby binding of transmitter to the receptor component initiates a chain-reaction through an associated G-protein that is transmitted to an effector component to effect change in the molecular environment.

The class of receptors known as G protein-linked receptors are typically characterized by a 7-helix organization, whereby the receptor protein is believed to traverse the membrane seven times. They also share a common signalling mechanism, whereby signal transduction across the membrane involves intracellular transducer elements known as G proteins. When a chemical messenger binds to a specific site on the extracellular surface of the receptor, the conformation of the receptor changes so that it can interact with and activate a G protein. This causes a molecule, guanosine diphosphate (GDP), that is bound to the surface of the G protein to be replaced by another molecule, guanosine triphosphate (GTP), triggering another conformational change in the G protein. When GTP is bound to its surface, the G protein regulates the activity of an effector. The effectors include enzymes such as adenylyl cyclase and phospholipase C; channels that are specific for calcium ions (Ca^{+2}), potassium ions (K^{+}), or sodium ions (Na^{+}); and certain transport proteins.

In general, activation of G protein-coupled receptors by transmitters will induce one or another of the following effector responses: activation of adenylyl cyclase, inhibition of adenylyl cyclase or stimulation of

phospholipase C activity. When the effector, adenylyl cyclase, is either activated or inhibited, it produces changes in the concentration of the molecule cyclic adenosine monophosphate (cAMP). Another effector, phospholipase C, causes one molecule of phosphatidylinositol-bisphosphate (PIP_2) to be cleaved into one molecule of inositol trisphosphate (IP_3) and diacylglycerol (DAG); IP_3 then causes calcium ions (Ca^{+2}) to be released into the cytoplasm. Alterations in cellular levels of cAMP and Ca^{+2} are two of the most important intracellular messages that in turn act to alter the behaviour of other target proteins in the cell.

Receptors can be classified based on which type of signalling pathway they activate in cells. This occurs at the level of the G proteins, which detect and direct signals from diverse receptors to the appropriate effector-response pathway. The three main groups of G proteins are: Gs-like, which mediate adenylyl cyclase activation; Gi-like, which mediate inhibition of adenylyl cyclase; and Gq-like, which mediate activation of phospholipase C.
Since one receptor can activate many G-proteins, the signal can be greatly amplified through this signal-transduction pathway.

A cell may contain multiple G proteins, each of which may interact with many different receptors and regulate several different effectors. When several receptors utilize a common G protein "transducer", this signal-transduction pathway allows for signal integration because many individual extracellular signals can be integrated to yield a cumulative intracellular signal such as elevated calcium levels. Thus, the G protein-linked receptor/effector systems generate complex networks of molecular interactions that allow for versatile regulation of inter-cellular communication and cell function.

A wide variety of chemical-messengers involved in regulating key functions in the body act through G protein-coupled receptors. These include neurotransmitters such as dopamine, acetylcholine and serotonin, hormones of the endocrine system such as somatostatin, glucagon and adrenocorticotropin, and lipid mediators such as prostaglandins and leukotrienes. Over one hundred different G protein-coupled receptors have been identified in the humans, and many more are expected be discovered. All of these receptors are believed to utilize one of the three principal G protein-effector signalling pathways (stimulation or inhibition of adenylyl cyclase or activation of phospholipase C).

In view of the diverse functions of G protein-coupled receptors in the body, it is not surprising that many existing therapeutic drugs act by directly modifying the function G protein-coupled receptors. In most cases, these drugs exert their effects on receptor function by binding to the same site on the receptor component as the natural chemical messenger. Such drugs can be classified into two types: 1) agonists, which mimic the action of natural transmitter by provoking activation of G protein-effector signalling pathways when they bind to the transmitter site; and 2) competitive antagonists, which block the binding of the transmitter by occupying the transmitter binding site but do not themselves activate G protein-effector pathways. A useful analogy is that of a lock and key, whereby agonists are different keys which can open the same receptor lock, while antagonists will block the key hole but will not open the lock. In a more general view, compounds which can bind to a specific region of the receptor are called ligands; agonists and antagonists are ligands which bind to the transmitter recognition site on the receptor.

Analysis of the effects of ligands n th ability f G protein-coupl d receptors to activate signalling pathways has sugg sted that the receptors exist in two f rms or "conformations", an "inactive" conformation which is silent and an "active" conformation which triggers G protein activation and effector signalling (Gilman, A.G., 1987, *Annu. Rev, Biochem.* 56: 615-649). Generally, ligands that can cause the receptor to assume the "active" conformation turn on signalling and are thus agonists. These compounds, at maximally effective concentrations, can elicit a full or partial response, and are termed full and partial agonists, respectively. Ligands that block or otherwise interfere with the interaction of agonists with the receptor, and thereby prevent agonists from activating the receptor, are known as competitive antagonists. These compounds are generally thought to act by binding to the transmitter site, but to have no intrinsic activity themselves (i.e. they do not turn on the signalling function of the receptor). Studies have shown that competitive antagonists can be further categorized into two classes, "neutral" antagonists which block agonist binding but have no effect on signalling, and inverse agonists (also known as negative antagonists) which can inhibit the "background" signalling of receptors in the absence of agonists.

This evidence has led to a model whereby active and inactive receptors co-exist in the cell in equilibrium, with agonists pushing the equilibrium to the active form, inverse agonists pushing it to the inactive form, and neutral antagonists blocking the chemical-messenger site while not favouring either conformation.

The experimental evidence underlying development of this model was primarily derived from two types of study. The first, conducted with animal tissues and receptor preparations therefrom, showed that receptor antagonists could decrease the agonist-independent activity of G

protein activation (Schultz, W., 1992, *supra*; Costa, T. t al., 1992, *Mol. Pharmacol.*, 41:549-560; C sta, T., and Herz., 1989, *Proc. Natl. Acad. Sci., USA*, 86:7321-7325; Murray R. and Keenan, A.K., 1989, *Cell Signalling* 1:173-179; Hilf, G. and Jakobs K.H., 1992, *Eur. J. Pharmacol.* 225:245-252). These observations showed that the receptor exhibited some activity in the absence of agonist which could be decreased by antagonists. From these studies, it was shown that some antagonists possess inverse agonist activity at G protein-coupled receptors, but that the level of this activity was so slight that it is difficult or impossible to measure experimentally.

The second source of evidence for inverse agonism comes from studies with mutated forms of G protein-coupled receptors. These receptor mutants, produced *in vitro* by genetic engineering of the receptor cDNA sequence, were found to show an unnaturally high level of spontaneous signalling activity when expressed in mammalian cells in culture. (Lefkowitz, et al., 1993, *Trends Pharmacol. Sci.* 14:303-307; Kjeinberg, M.A. et al., 1992, *J. Biol. Chem.*, 267:1490-1433; Samama, P., 1993, *J. Biol. Chem.*, 268:4625-4636; Ren, Q., 1993, *J. Biol. Chem.*, 265:16483-16457). In these cases, the elevated spontaneous activity of the receptor was measured at the level of effector signalling, as increases in cAMP or IP_3 second messengers. The mutations appear to change the equilibrium of inactive and active conformations, resulting in a higher basal level of the active form. The ability of an antagonist to decrease the spontaneous activity of the active receptor mutant was consistent with inverse agonist action of the antagonist.

The ability to detect inverse agonist activity of receptor ligands clearly depends on the ability to measure spontaneous, agonist-independent receptor coupling to cell signalling pathways. While some agonist-independent activity can be observed with receptors from natural

sources, the levels of activity are too low to permit reliable measurements. Conversely, mutated receptors, expressed from cloned genes in cultured cells, have been fundamentally changed by mutation to exhibit aberrant behaviour which does not reflect the behaviour of the natural receptor. Hence, prior to development of the present invention, a suitable assay system to perform such testing did not exist.

The development of a method of testing compounds for their abilities to inhibit the agonist-independent activity of G protein-coupled receptors has great utility for many industries whose goal is to develop chemical substances that interact with G protein-coupled receptors. Since G protein-coupled receptors are ubiquitous and widely used in nature to transmit cellular signals, this invention has utility for different industries including: the pharmaceutical industry; the pest-control industry; the aquaculture industry; the food industry; and the fragrance industry.

The pharmaceutical is particularly interested in the potential therapeutic applications of inverse agonists in pathologies associated with spontaneous receptor activity. There are several genetic diseases shown to be associated with mutations in G protein-coupled receptors resulting in constitutive receptor signalling. Drugs which possess inverse agonist activity could have particular therapeutic relevance for such conditions.

Current methods of screening compounds for their ability to interact with G protein-linked receptors are well known in the art but are less than adequate. For example, one type of assay, termed a ligand binding assay, measures the ability of a compound to bind to the transmitter recognition site on the receptor. In its most widely-applied form, the assay involves incubating an

aqu ous suspension f the r cept r (gen rally a cell membrane preparation containing th rec ptor) with a radioactiv derivativ of a ligand known t bind t the transmitter site. By measuring the amount of radioactive ligand bound in the presence of the test compound, it is possible to detect ligands for the same site by the inhibition of radio-ligand binding to the receptor. This type of assay, and variations based on alternative labels (e.g. fluorescent ligands) or technical improvements to facilitate automation (e.g. scintillation proximity assay) are well known in the art and are frequently the first screening test carried out to identify compounds which interact with receptors, meriting further examination as potential therapeutic candidates.

Other types of assays which are generally used to further investigate compound activity in the ligand binding assay, permit the discrimination of agonist from antagonist ligands. However, these two drug classes are not reliably distinguishable in the ligand binding assay, and require systems which can measure receptor activation of G protein-effector signalling activities. Such assays are generally termed bioassays, and were traditionally carried out with animal tissues in organ baths where the activity of the receptor could be measured as a complex response of the tissue (e.g. muscle contraction). In these preparations, agonists are identified by their ability to activate the tissue response, while antagonists do not activate the response themselves but competitively block the activation by agonists. Variations on this screening procedure use biochemical assays, such as the production of the cAMP or IP_3, to measure the effects of compounds on receptor coupling to effector response pathways. They are well known in the art.

In addition to discriminating agonists from antagonists, these bioassay systems also permit more

detailed analysis of the properties of the drug candidate, such as potency (the concentration at which the ligand exerts its effect on receptor signalling), as well as efficacy (the maximal effect of the drug on receptor activity). The measurement of ligand efficacy permits further classification of agonist ligands into full agonists and partial agonists, the latter group producing only a partial response even at maximal concentrations. In these assays, antagonists have zero efficacy (i.e. they produce no activation of the response pathway). These parameters permit selection of the most promising drug candidates, based on their ranking in the different assays, for further development and analyses in more complex models (e.g. animal models of disease).

With the enormous progress in the cloning, sequencing and expression of genes which encode G protein-coupled receptors, (and drug target proteins in general), there has been a major shift away from animal tissues over to recombinant receptors, the term used to describe receptors which are produced by expression of the cloned gene in cultured cells. Such recombinant receptors are now the principal source of receptors for drug screening in ligand binding assays and in bioassays (in cases where the receptor cDNA has been cloned). The use of recombinant receptors demonstrates many advantages over tissue sources, including the ability to use human receptors expressed from human genes, the facility with which large amounts of the protein can be produced, the fact that a single receptor subtype (subtypes are closely related but distinct receptors which use the same natural transmitter) can be tested and compared with closely related subtypes, etc.

Bioassay systems that are known in the art for recombinant G protein-coupled receptors are based on the ability of the expressed receptor to activate endogenous signalling pathways in the host cell. The first assays

measur d th activity of effectors (adenylyl cyclas and phospholipas C) utilizing known biochemical assays from tissu -bas d assays. Thes gen rally employ mammalian cell lines which have been made to express the cloned receptor DNA using techniques (transfection, transformation) which are well known to and routinely practised by technicians trained in the art. One example uses fluorescent dyes sensitive to the concentrations of specific ions, primarily calcium, to measure changes in the intracellular ion concentrations associated with activation of receptors coupled to Gq-phopsholipase C signalling. An increasing number of new systems involve genetic engineering of the host cell to facilitate measurement of the effector response to receptor activation. In one example, the gene for an enzyme that is readily assayed, such as beta-galactosides, is inserted into the host cell genome under the control of a promoter element sensitive to cAMP levels. Receptors which activate adenylyl cyclase and thereby increase cAMP levels in these cells will activate expression of the beta-galactosidase "reporter" gene. Measurement of the enzyme activity in a simple assay thus provides a measure of receptor activity.

In general terms, all of these bioassay systems are designed for and well-adapted to the task of discriminating ligands which activate the receptor (i.e. agonists) from ligands which do not activate the receptor (i.e. antagonists).

Finally, inverse agonist activity of G protein-coupled receptor antagonists is known to exist. However, screening assays suitable for the routine testing of ligands for inverse agonism had not been described prior to the present invention. Hence, the need for development of an assay or screening system which permits the testing of compounds for their ability to decrease agonist-independent or spontaneous activity. The present invention provides one

skill d in th art with the ability to reliably measur spontaneous r c pt r activity nec ssary t obtain a window of agonist-indep ndent activity in which to bserv inverse agonist effects. None of the *in vitro* screening systems currently known can sufficiently quantify inverse agonist activity of ligands and rank compounds based on this activity. The present invention, therefore, provides an improved method for testing and ranking compounds for their abilities to inhibit the agonist-independent activity of G-protein linked receptors, without introducing mutations into the receptor sequence.

SUMMARY

It is an object of the present invention to provide a method for the testing and/or screening of chemical compounds for their abilities to inhibit the agonist-independent activity of G protein-coupled receptors, and to rank such compounds based on their relative activities to inhibit such activity. The method involves: 1) expressing the cloned cDNA encoding the G protein-coupled receptor in a cell expression system in such a manner as to generate a reproducible "window of chemical-messenger-independent activity" (hereinafter referred to as "activity window") that allows for the discrimination of chemical compounds based on their relative activities to inhibit chemical-messenger-independent activity; 2) measuring the agonist-independent activity of the receptor in whole cells or membrane fragments containing G protein, an appropriate effector, and cloned G protein-linked receptor, using biochemical or other assay procedures which permit quantification of G protein-effector response pathways in the system; 3) contacting the compound of interest with the system under conditions permitting interaction of the compound with the receptor; and 4) determining whether and to what degree the compound affects the agonist-independent

activity of the rec pt r. It is intended that the method be suitabl for t sting inverse fficaci s of compounds against any G prot in-coupled rec pt rs f r which th cloned cDNA has been isolated or will be isolated in the future.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Activity of adenylyl cyclase in membranes from uninfected Sf9 cells and Sf9 cells infected for 48 hr with either wild-type (*wt*) baculovirus or recombinant baculovirus encoding the human ßAR. The production of cAMP was measured in the absence (□) or presence of 10 μM isoproterenol (▨). The data shown represent means from at least three experiments carried out in duplicate. *Inset*, the production of cAMP was measured in membranes from cells that had been deprived of serum for the final 18 hr of infection and treated with 1 μM alprenolol for 30 min. before harvesting.

Figure 2. Effect of receptor level on GTP-, isoproterenol-, and forskolin-stimulated adenylyl cyclase activity. The level of βAR in membranes was varied by infecting Sf9 cells for 48 hr at increasing multiplicities on infection, and receptor levels were determined as described in Materials and Methods. Production of cAMP was measured in the presence of 53 μM GTP alone (■) or with the additional inclusion of 10 μM isoproterenol or 100 μM forskolin. The net stimulation by isoproterenol (□) and forskolin (●) was determined by subtracting the level of activity observed in the presence of GTP only, at each concentration of receptor.

Figure 3. Effect of alprenolol on βAR-stimulated adenylyl cyclase activity. The production of cAMP in membranes from Sf9 cells lacking βAR (i.e., either uninfected or infected with wile-type baculovirus) was subtracted from that in membranes expressing the receptor, in the presence of isoproterenol (10 μM), alprenolol (10μM), or neither. The data shown represent means from four experiments.

Figur 4. Inhibiti n of agonist-ind pendent βAR-stimulat d ad nylyl cyclase by alpr nolol in membranes from cells d priv d of s rum. Sf9 c lls infected with baculovirus and cultured in serum-free medium for the final 18 hr of infection were treated with vehicle (O) or 1 μM alprenolol (◊) for 30 min before membrane preparation. The production of cAMP was measured at the concentrations of alprenolol shown on the *abscissa*. The data shown are from a representative experiment (two determinations) and were normalized to the fitted asymptotes, which were as follows: estimated cAMP production in the absence of alprenolol ($y_{x=0}$) was 45 ± 1 and 75 ± 1 pmol/mg/min in alprenolol-treated and control membranes, respectively, and that in the presence of a maximally inhibiting amount of alprenolol ($y_{x=\infty}$) was 30 ± 1 and 34 ± 1 pmol/mg/min in alprenolol-treated and control membranes, respectively.

Figure 5. Effect of time and infection on the inhibition of membrane adenylyl cyclase activity by propanolol. Sf9 cells were infected with baculovirus encoding the βAR for 0(▲), 12 (◊), 24 (O), or 48 hr (□) before preparation of membranes, and the production of cAMP was measured at the concentrations of propranolol indicated on the abscissa. The estimated membrane concentrations of β_2AR in this experiment were 0.04, 1.9, and 10.0 pmol/mg protein at 12, 24, and 48 hr of infection, respectively. The data shown are representative of two experiments, and a comparable pattern was observed with alprenolol.

Figure 6. Inhibition of agonist-independent adenylyl cyclase activity in membranes from Sf9 cells expressing β_2AR. Production of cAMP was measured in the presence of dichloroisoproterenol (♦), labetalol (♦), pindolol (●), or timolol (O), at the concentrations indicated on the *abscissa*. The representative data shown were obtained on the same day with the same membrane preparation and were included in the simultaneous analyses for the determination

f th EC_{50} and E_{inv} valu s listed in Table 1. Isopr terenol (□) was included as a positiv control. For th curv s shown, the data w re r analys d with $y_{x=0}$ common to all fiv sets of data.

Figure 7. Effect of agents with low E_{inv} on inhibition of adenylyl cyclase activity by timolol. A, Proportion of adenylyl cyclase activity associated with β_2AR expression observed in the presence of 10nM timolol, 10 μM labetalol, or both. The results shown represent means from two experiments. B, Effect of timolol on the production of cAMP in membranes from Sf9 cells expressing βAR, in the absence (○) and presence (◇) of 10 μM dichloroisoproterenol. The data shown are representative of two experiments.

Figure 8. Characterization of 5HT-2C receptors in intact Sf9 cells. Inset: Time course of receptor expression: Sf9 cells infected with the 5HT-2C baculovirus were harvested at various times post-infection and the number of [^{3}H]mesulergine binding sites on intact cells measured as described in Methods. Results are expressed as thousands of mesulergine binding sites per cell (mean ± S.E.M. for three experiments. Main Figure: Sf9 cells were infected with the 5HT-2C baculovirus and the production of total [^{3}H]-inositol phosphates measured at various times post-infection (12 hr, 20 hr, 38 hr and 72 hr) under several conditions (see Methods). For each time point, IP production was measured: under basal conditions, i.e. no added drugs (□); in the presence of 10 μm serotonin (■); in the presence of 10 μm serotonin plus 10 μm mianserine (▨); and in the presence of 10 μm mianserine alone (▩). The same tests were also carried out at 38 hr post-infection in cells infected with the wild type baculovirus (Wile Type). Results are expressed as dpm of total [^{3}H]inositol phosphates released over 20 min assay (mean ± S.E.M. for triplicate samples in one representative experiment).

Figur 9. Dose-response curv s for ag nist-stimulati n of IP production by the rec pt r in Sf9 cells. Sf9 c lls inf cted with th 5HT-2C baculovirus wer assayed f r t tal [^{3}H] inositol phosphate production at 20 hr (see Methods) in the presence of increasing concentrations of the agonist drugs D.O.I. (○), 5HT (▲) and RU24969 (△). Results are expressed as % increase of IP production over that measured in the absence of added drug (mean of three separate experiments).

Figure 10. Inhibition of basal IP production by serotonergic antagonists. Sf9 cells were infected with the 5HT-2C baculovirus and IP production measured (see Methods) at 38 hr post-infection in the presence of increasing concentrations of various drugs. The results are expressed as the % inhibition of basal IP production (mean for three separate experiments in triplicate). Calculated values for drug potencies and maximal effects are presented in Table 1.

Figure 11. Competitive effects of spiroxatrine on agonist-stimulation and antagonist-inhibition of basal IP production. Top Panel: Sf9 cells were infected with the 5HT-2C baculovirus and total IP production assayed (see Methods) at 20 hr post-infection in the presence of 5HT (1 μm) and increasing concentrations of spiroxatrine. The results are expressed as % of basal IP production, and represent the mean of three experiments. The EC_{50} value for spiroxatrine inhibition calculated from the data is shown in Table 1. Bottom Panel: Sf9 cells infected with the 5HT-2C baculovirus were assayed for total IP production (see Methods) at 38 hr post-infection in the presence of increasing concentrations of mianserine alone or with 10 μm spiroxatrine. The results represent the mean of three separate experiments carried out in triplicate.

DETAILED DESCRIPTION

The following definitions of t rms are used to describe the invention.

A "window of chemical-messenger-independent activity" is used synonymously with a "window of agonist-independent activity" and/or a "window of spontaneous activity", and all are defined as the range of spontaneous receptor activity that results when the appropriate concentration of a G protein-coupled receptor is attained through expression of cloned cDNA in cultured cells, to allow for the ranking of chemical-messengers on the basis of their abilities to inhibit the spontaneous activity of said G-protein linked receptors.

A "chemical-messenger" is defined as any messenger, in the absolute broadest sense, natural or unnatural, that induces an effect or blocks an effect on a proteinaceous receptor, including chemical substance transmitters (eg. as neurotransmitters, hormones, and lipid mediators such as prostaglandins and leukotrienes; usually act on chemoreceptors), light (eg. signalling via a rhodopsin receptor; usually act on electromagnetic (photo) receptors), and stimuli such as temperature and mechanical signals (eg. physical or chemical damage, vibration, touch, pressure, movement) which act on mechanoreceptors, thermoreceptors and nociceptors.

An "effector protein" is defined as any protein which is activated or inactivated by a G protein. Some Examples of effector proteins include adenylyl cyclase and phospholipase C.

A "G protein" is defined as any member of the family of signal transducing guanine nucleotide binding proteins.

A "G protein-coupled receptor" is defined to be any cell surface transmembrane protein, that when activated by a chemical, in turn activates a heterotrimeric guanine nucleotide-binding protein (G protein).

A "ligand" is intended to include any substance that interacts with a receptor. It may stimulate, inhibit, or cause some effect for the activity of the receptor. An "agonist" is defined as a ligand increasing the functional activity of a receptor (ie. signal transduction through the receptor). An "competitive antagonist" is defined as a ligand which can bind to the transmitter recognition site on the receptor and thereby block receptor activation by agonists. An inverse agonist is defined as a ligand which can decrease the spontaneous activity of the receptor..

A "receptor" is intended to include any molecule present inside or on the surface of a cell, which molecule may effect cellular physiology when either stimulated or inhibited by a ligand. A "G protein-linked receptor" is intended to include any receptor that mediates signal transduction by coupling with a guanine nucleotide binding protein.

A "second messenger" is defined as an intermediate compound whose concentration, either intercellularly or within the surrounding cell membrane, is raised or lowered as a consequence of the activity of an effector protein. Some examples of second messengers include cyclic adenosine monophosphate (cAMP), phosphotidyl inositol (PI), such as inositol triphosphate (IP_3), diacylglycerol (DAG), calcium (Ca^{+2}) and arachidonic acid derivatives.

A "test substance" is intended to include any drug, compound or molecule with potential biological activity.

Accordingly, the present invention relates to a method of testing compounds for their abilities to inhibit chemical-messenger-independent activity of G protein-coupled receptors. The ability to discriminate relative abilities of such inhibitory activity is based on the ability to generate a window of chemical-messenger-independent activity (an "activity window") that, previous to this invention, was either unknown and/or unattainable in the art. This method is applicable to any G protein-coupled receptor once a cDNA clone for that receptor has been generated, including so-called "orphan-receptors" for which no known agonist has yet been identified. This invention involves a method of testing chemical compounds for their abilities to inhibit chemical-messenger-independent activity of G protein-coupled receptors involving: expressing DNA encoding a G protein-coupled receptor in a cell expression system in such a manner as to generate a reproducible "window of chemical-messenger-independent activity" that allows for discrimination of chemical compounds based on relative ability to inhibit chemical-messenger-independent activity of said G protein-coupled receptor; measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-independent activity of said receptor in said system comprising whole cells or membrane fragments containing G protein, an appropriate effector, and cloned G protein-linked receptor; contacting a test-compound with the system under conditions permitting interaction of the test-compound with said receptor; and measuring the change, if any, of the quantifiable parameter which reflects the ability of the test compound to inhibit the chemical-messenger-independent activity of the G protein-coupled receptor.

In another embodiment, this invention involves a test kit including: whole cells or membrane fragments thereof, comprising cloned G protein-coupled receptors, functionally

xpr ssed at a conc ntration demonstrating a "window of chemical-mess ng r-indep ndent activity"; appr priat reagents; and supporting documentati n enabling th working of this invention.

In yet another embodiment, the method comprises: i) expressing a G protein-coupled receptor gene in a cell-line using a baculovirus expression system in Sf9 cells; ii) adding a test substance to the whole cells or to membrane fragments prepared from cell lysates; and iii) assaying for the amount of cyclic AMP produced either in whole cells or in membrane fragments.

In a further embodiment, the method comprises: i) expressing a G protein-coupled receptor gene in an Sf9 cell; ii) adding a test substance to the whole cells or to membrane fragments prepared from whole cell lysates; and iii) assaying for the amount of inositol phosphate produced in whole cells or in membrane fragments.

In additional embodiments of the invention, the method comprises: i) expressing the G protein-coupled receptor gene in an insect cell expression system; ii) adding a test substance to whole cells or membrane fragments prepared from whole cell lysates; and iii) measuring any quantifiable parameter which reflects the receptor activation of G protein-effector response pathways, including G protein activation (e.g. by GTP binding or GTPase activities) and cellular responses such as intracellular calcium mobilization.

This invention describes a test system that differs from those in the prior art in that it is based on the discovery that G protein-linked receptors, when expressed at relatively high levels, demonstrate a range (or window) of elevated levels of chemical-messenger-independent activity, that enables detection and reproducible ranking

f chemical compounds on th basis f ability to inhibit such activity. Thus, this method is dependent upon cr ating a window of chemical-messeng r-ind p nd nt activity of a G protein-coupled receptor within which inhibitory activity can be detected and reproducibly measured.

Generation of a Window of Chemical-Messenger-Independent Activity

Following infection of an appropriate cell expression system with cDNA coding for a G protein-coupled receptor (as described below), the level of second messenger molecules (eg. cAMP or IP_3), sensitive to chemical-messenger-independent activity of the functionally expressed G protein-coupled receptor, are repeatedly measured. When these measurements demonstrate a rise in the chemical-messenger-independent activity in direct response to the level of the cloned receptor expressed in a functional manner, they can be used to define an activity window dependant on time after viral infection within which signal detection is sufficiently high to rank compound activity for that receptor. This activity window is directly related to the level of receptor expression and reflects a specific range of receptor concentrations: concentrations that are too low will not allow for ample signal detection to rank compounds on a statistically significant basis, whereas concentrations that are too high either reach maximal levels, and/or are toxic to the cultured cell and generate variable results. These activity windows are reproducibly attainable at a specified time measured post-infection, enabling repeated reproduction of a defined test system by stopping gene expression at a the specified time following infection.

Demonstration of generation and use of an activity window are presented within the examples described in

herein. In these embodiments, it was possible to demonstrate a time-dependent rise in the spontaneous activity of the receptors which could be inhibited in a dose-dependent manner by test compounds such as antagonist ligands. By defining a time-window following viral infection in this manner, the assay proves to be highly reproducible and to permit fine discrimination of ligands based on the potency of inhibitory activity (ie. the "EC_{50}", the concentration necessary to evoke a response that is half the maximum possible), the affinity of the compound for the receptor (ie. the "K_d"), and the inverse efficacy (ie. "E_{inv}" , the fraction of spontaneous receptor activity that can be inhibited by a given compound), also described as the relative ability of the ligand to produce a maximal inhibition of spontaneous activity.

A clear demonstration of a continuum of inverse agonism from neutral to partial to full inverse agonists mirrors the classification of agonists as partial and full. The ability to quantitate inverse efficacies for G protein-coupled receptor ligands, as provided by the present invention and described in the examples permits demonstration of partial inverse agonism for two such receptors (β2-adrenergic and 5HT-2C serotonergic receptors) coupled to two different G protein-effector pathways (adenylyl cyclase and phospholipase C, respectively) in addition to permitting quantitative and reproducible ranking of ligands based on their inverse efficacies. This activity window can also be used to measure the ISA of a compound, which is a useful clinical parameter of drug action that has relevance to therapeutic and is a measurement well known in the art.

Finally, as described in the attached publication, a significant correlation was observed between the ranking of ligands in the assay as inverse agonist of the human β2-adrenergic receptor, and a clinical parameter of drug

action (ISA) which has relevance to therapeutic activity of these ligands as cardiovascular drugs. The assay, based on expression of G protein-coupled receptors at high levels in cultured insect cells, thus appears to have value for predicting parameters of drug action on the natural receptor in the body.

Several lines of evidence indicate that the spontaneous activity of G protein-coupled receptors expressed in the baculovirus-insect cell system is not an artifact of the expression system or cellular environment, but rather an amplification of intrinsic spontaneous activity not normally detected in other expression hosts which produce low levels of receptors compered to the baculovirus system.

The preliminary step of the assay methods of the present invention is to express exogenous cDNA in Sf9 cells. For clarity the ß-adrenergic receptor is used as an example, and the 5HT-2C receptor is used as another example. However, the descriptions apply equally well to any G protein-linked receptor whose coding DNA has been cloned, such as thyrotropin, lutropin-choriogonadotropin, dopamine and histamine receptors, etc. Likewise, once clones are available for other G protein-coupled receptors, they can also be used in the present invention as described below. Even chemical messengers demonstrating activity for so-called "orphan receptors", for which no natural agonist ligand has yet been identified, can be detected and ranked using this invention because it is based on detecting results of cognate second messenger pathways.

Creation of Recombinant Vector and Infection into Cell-line

The cDNA coding for functional G protein-coupled receptor can be inserted into any suitable vector, cloned,

and expr ssed in an appr priat cell line which will be able t express and proc ss th receptor, in addition t possessing th necessary biochemical machin ry to respond t signal transduction through a given r ceptor. Transfection may be perf rmed according to known methods. In general, a DNA sequence encoding a receptor may conveniently be subjected to recombinant DNA procedures. The vector may be an autonomously replicating vector, such as a plasmid, or the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The DNA sequence encoding the receptor should be operably connected to a suitable promoter sequence, a suitable terminator sequence and may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. The procedures used to ligate the DNA sequences coding for the receptor, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Cells which may be used in the present method are cells which are able to express the cloned receptor at a sufficiently high level to generate an activity window, in addition possessing the ability to respond to signal transduction through a given receptor by cellular growth. Such cells are typically eukaryotic cells, such as mammalian, or insect cells. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are well known to persons skilled in the art. (Kaufman and Sharp, 1982, *J. Mol. Biol.*, 159:601-621;

Southern and Berg, 1982, *J. Mol. Appl. Gen t.*, 1:327-341; Loyter t al., 1982, *Proc. Natl. Acad. Sci. USA*, 79:422-426; Wigl r t al., 1978, *Cell*, 14:725; Corsaro and P arson, 1981, *Somatic Cell Gen tics*, 7:603; Graham and van der Eb, 1973, *Virology*, 52:456; Neumann et al., 1982, *EMBO J.*, 1:841-845; and Wigler et al., 1977, *Cell*, 11:223-232).

One such vector and expression system that works particularly well with this invention entails constructing a recombinant baculovirus expression vector, capable of expressing a G protein-coupled receptor in a host insect cell line (eg. Sf9 cells). Examples of how to construct suitable recombinant baculovirus vectors are described in U.S. Patent No. 4,745,051, Smith G.E., and Summers, M.D., 1988, and U.S. Patent No. 4,879,236, Smith G.E., and Summers, M.D., 1989. The general procedures of recombinant DNA technology pertaining to creation and manipulation of the baculovirus system are well known to those skilled in the art (O'Reilly, D.R., et al., 1992, *Baculovirus Expression Vectors, A laboratory Manual*, (New York: W.H. Freeman and Company; Davies, A.H., 1994, Bio/Technology, 12:47-50).

One embodiment of this invention describes and demonstrates the expression of a G protein-coupled receptor at high levels in a baculovirus-insect cell system. The *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) has been shown to be suitable as a viral expression vector for the efficient production in cultured insect Sf9 cells of mammalian membrane proteins from foreign genes (Luckow, V.A., and Summers, M.D., 1988, *Bio/Technology*, 6:47-55; Miller, L.K., 1988, *Annu Rev. Microbiol.*, 42:177-199). A number of G protein-coupled receptors have been expressed in the baculovirus-Sf9 cell expression system, and were found to maintain binding properties characteristic of the "natural" receptors in tissues or expressed from cloned cDNAs in mammalian cell lines

(Mouillac, t al., 1992, *supra*; Park r, E.M., 1991, *J. Biol. Chem.*, 266:519-527; Wong, S.K.F., 1990, *J. Biol. Chem.*, 265:6219-6224).

One method of preparing a recombinant expression vector containing cDNA coding for a G-protein-coupled receptor, described generally, involves cleaving baculovirus DNA to produce a DNA fragment comprising a polyhedrin gene or portion thereof, including a polyhedrin promoter. A recombinant shuttle vector is prepared by inserting the baculovirus DNA fragment into a cloning vehicle and thereafter inserting a selected gene coding for a G protein-coupled receptor into the thus modified cloning vehicle such that it is under the transcriptional control of the polyhedrin promoter. The resultant recombinant baculovirus is then used to infect susceptible insects or cultured insect cells and the protein product from the incorporated selected gene is produced from the infection.

One particular embodiment of the invention, wherein βAR receptors are expressed within a baculovirus expression system in Sf9 cells, indicates that the interactions between βAR ligands and the β_2AR using this expression system accurately mimic the process of βAR transduction in mammalian systems. βAR expressed in Sf9 cells exhibits normal pharmacological properties and undergo phosphorylation and palmitoylation in the expected manner (Mouillac, B., 1992, *J. Biol. Chem.*, 267:21733-21737; Parker, E.M., et al., 1991, *J. Biol. Chem.*, 266:519-527). Furthermore, the binding affinities of antagonists obtained in the present invention are consistent with those reported in membranes prepared from βAR-expressing mammalian cells (Samama, P., et al., 1993, *J. Biol. Chem.*, 268:4625-4636; Jasper, J.R., et al., 1988, *FASEB J.*, 2:823-828) and tissues (Bevilacqua, M. et al., 1988, *J. Cardiovasc. Pharmacol.*, 11(Suppl. 2):S25-S27; Riva, E., et al., 1991,

Br. J. Pharmacol., 104:823-828; Minneman, K.P., et al., 1979, *Mol. Pharmac l.*, 16:1979).

Preparation of Whole Cells or Membranes Containing the G Protein-linked Receptor, the G Protein, and the Effector

When functional receptor expression reaches a sufficiently high density to create an activity window, the cells are harvested and washed with an appropriate buffered solution to arrest expression activity. If membranes, rather than whole cells are to be used in the test system, cells can be lysed in solution containing appropriate proteolytic enzyme inhibitors. The resulting membrane fragments are washed and the protein content determined by standard, well-known techniques.

Compound Screening

The chemical-messenger-independent activity is measured by any suitable quantifiable parameter using biochemical or other assay procedures that indicate the agonist-independent activity of the cloned receptor in the expression system comprising whole cells or membrane fragments containing G protein, an appropriate effector, and cloned G protein-linked receptor. Many appropriate biochemical or other assay procedures, such as measuring the activity of effectors such as adenylyl cyclase or phospholipase C activity are determined by standard techniques that are well known to persons skilled in the art.

For example, one method of determining the activity of the effector is by measuring levels of second messenger such as c-AMP (for eg., Gilman, A.G., 1970, *Proc. Natl. Acad. Sci., USA*, 67:305-312) or inositol phosphate

(Fargin, A., et al., 1989, J. Biol. Chem., 254:14818-14852).

Once the basal level of chemical-messenger-independent activity is measured, the test substance is incubated with the whole cells or with membrane fragments comprising the cloned G protein-coupled receptor, G protein, and effector, under conditions allowing for compound interaction with the cloned receptor. Finally, the resulting receptor activity level is measured and analyzed.

The present invention is described in further detail in the following non-limiting Examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

Example 1: ß-Adrenergic Receptors (ß-AR)

Construction of $ß_2$-AR Recombinant Baculovirus

The plasmid vector pTZ18R (Pharmacia LKB Biotechnology Inc.) containing the entire $ß_2$-AR coding sequence cloned in the *Eco*RI-*Hind*III sites (Kobilka, B.K., et al., 1987, J. Biol. Chem., 262:15796-15802), was digested by *Eco*RI-*Hind*III. The resulting fragment containing the coding sequence for $ß_2$-AR was isolated and the 5' protruding ends were filled in with Klenow DNA polymerase. A plasmid derived from the pJVNheI baculovirus expression vector (Vialard, J., et al., 1990, *J. Virol.*, 64:37-50) and containing the ß-galactosidase gene was digested by NheI

and the 5′ protruding ends wer also filled in with Kl now DNA polymerase. Th n fragment coding f r β_2-AR was then insert d in th pJVNheI-derived vect r by blunt-end ligation and the orientation was verified by restriction endonuclease digestion (PstI). Transfer of the β_2-AR coding sequence and of the ß-galactosidase gene to the AcNPV genome was achieved by cotransfection in Sf9 cells using the calcium phosphate precipitation procedure (Summers, M.D., and Smith, G.E., 1987, *Tex Agric Exp. Stn. Bull.*, 1555:10-39). Recombinant baculovirus were purified by successive plaque assays using ß-galactosidase detection as described (Vialard, J., 1990, *supra*). Stocks of baculovirus containing the β_2-AR coding sequence were amplified and used to express β_2-AR in Sf9 cells.

Cell culture and infection with recombinant baculovirus.

Sf9 cells were cultured at 27 °C in Grace's supplemented insect medium (GIBCO), to which were added 10% fetal bovine serum (P.A. Biologicals, Sydney, Australia), 0.1% Pluronic F-68 (GIBCO), 50 µg/ml gentamicin sulfate, and 2.5 µg/ml fungizone. For some experiments, serum was omitted from the culture medium for the final 18 hr of infection. For most experiments, cells were grown in 100-ml spinner flasks; in some cases, cells were grown as monolayers in 75-cm^2 flasks. Cells were infected with wild-type or recombinant baculovirus when they reached a density of 1.0-4.0 x 10^6 cells/ml and were harvested 48 hr after infection, unless indicated otherwise. Experiments were carried out either on preparations of washed membranes or on whole cells.

Preparation f washed membranes from Sf9 cells.

Washed membranes were prepared essentially as described previously (Mouillac et al., 1992, *J. Biol Chem.*, 267:21733-21737). Infected cells (10 ml or 20 ml) were washed twice with 2 volumes of ice-cold phosphate-buffered saline and lysed with a Polytron homogenizer (two 5-sec. bursts) in 10 ml of cold buffer containing 5 mM Tris-HCl, pH 7.4, 2mM EDTA, 10 μg/ml benzamidine, 5μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin. Lysates were centrifuged at 500 x g for 5 min. at 4 °C, and the pellet was washed twice in 10 ml of buffer. The membranes were resuspended in buffer containing 75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 2mM EDTA, 10 μg/ml benzamidine, 5μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin, and were then used for adenylyl cyclase or binding assays. Protein content was determined according to the method of Bradford (Bradford, M.M., 1976, *Anal. Biochem*, 72:248-254).

Assay of adenylyl cyclase activity.

In membranes, assays were carried out using 5-10 μg of protein with 0.12 mM ATP, 1-2 x 10^6 cpm/assay tube of [α-^{32}P]ATP, 0.10 mM cAMP, 53 μM GTP, 2.7 mM phospho(enol)pyruvate, 1.0 IU of myokinase, 0.2 IU of pyruvate kinase, 4 μg/ml benzamidine, 2 μg/ml soybean trypsin inhibitor, 2μg/ml leupeptin, and varying concentrations of AR ligands, in a total volume of 50 μl. Samples were incubated at 37 °C for 15 min. or 30 min., and the reaction was terminated by addition of 1 ml of a cold solution containing 0.3 mM cAMP, 20,000 cpm of [^{3}H]cAMP, and 0.4 mM ATP. [^{32}P]cAMP was separated by chromatography using a Dowex gel, followed by aluminium oxide (Salomon et al., 1974, *Anal. Biochem.*, 58:541-548).

Assay f intr cellular cAMP.

Intracellular cAMP was determined by competition with [^{3}H]cAMP for a specific binding protein (Gilman A.G., 1970, *Proc. Natl. Acad. Sci. USA*, 67:305-312). Cells that had been infected for 48 hr with either wild-type or recombinant baculovirus encoding the human β_2AR were deprived of serum for the final 18 hr of infection and then used for experiments. One-milliliter aliquots containing 4 x 10^6 to 1 x 10^7 cells were treated for 30 min. at ambient temperature with various ligands in serum-free medium. cAMP was recovered from the cells as described previously (Insel, P.A., and Sanda, M., 1979, *J. Biol Chem.*, 254:6554-6559) Amersham kit no.1 TRK.432). One sample was prepared for each experimental condition, and each was assayed in duplicate for cAMP. Cellular cAMP was estimated by combining 50 μl of supernatant with [^{3}H]cAMP (4.5 nM final concentration) and allowing the labelled and unlabelled forms of the nucleotide to compete for a high affinity cAMP-binding protein for 2 hr at 4 °C; unbound [^{3}H]cAMP was removed by adsorption to charcoal followed by centrifugation, and the amount of radioactivity from the supernatant was determined. The amount of cAMP in each aliquot was estimated from a standard curve determined with samples that ranged from 1 to 16 pmol of cAMP/assay tube.

Binding of Adrenergic Receptor liganda.

Washed membranes were labelled with ^{125}I-CYP to study the binding properties of the β_2AR. For determinations of capacity, membranes were incubated with a saturating concentration of the radio-ligand (≥300 pM; K_d = 33 pM) in the absence or presence of 10^{-5} M alprenolol, with the difference in bound ^{125}I-CYP between the two conditions being

taken as the total specific binding. The inhibition of binding of a substrating concentration of ^{125}I-CYP (169 ± 8 pM) by increasing concentrations of unlabelled AR ligands was used to estimate the binding affinities of the latter compounds. In each case, assays were carried out in triplicate and membranes were incubated for 1.5 hr at ambient temperature in buffer containing 75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 5 mM EDTA, 10 µg/ml benzamidine, 5 µg/ml soybean trypsin inhibitor, and 5 µg/ml leupeptin. Unbound radio-ligand was removed by rapid filtration through Whatman GF/C filters, which were then rinsed three times with 2 ml of 25 mM Tris, pH 7.4, at 4 °C.

Analysis of Results

The concentration dependence of adenylyl cyclase activity on various AR ligands in membranes was analyzed according to a four-parameter logistic equation analogous to the Hill equation (ALLFIT) (De Lean et al., 1978, *Am. J. Physiol.*, 235:E97-E102). For all analyses, the slope factor was fixed at 1. For each βAR ligand listed in Table 1, replicate experiments were fitted simultaneously with ED_{50} common to all sets of data, allowing the asymptotes (i.e., $y_{x=0}$ and $y_{x=\infty}$ to vary for individual sets of data. The fitted values of $y_{x=0}$ and $y_{x=\infty}$ were used to define a_s and a_i, respectively, for the calculation of E_{inv} (see below). The inhibition of ^{125}I-CYP binding by βAR antagonists was analyzed according to the multi-site model by nonlinear least-squares regression, as described (De Lean, A. et al., 1982, *Mol. Pharmacol.*, 21:5-16). Data were analyzed assuming a single class of binding sites. For each unlabelled ligand, data from all experiments were fitted simultaneously with the affinity (K_d) common to all sets of data. Estimates of nonspecific binding and total receptor concentration were allowed to vary among individual sets of data. Unless specified otherwise, values listed in the text, table, and figure legends represent averages ±

standard errors, and such values were compared were appropriate using Student's t test; for fitted values of K_d EC_{50}, y_{max} and y_{min}, the accompanying error reflects the range over which the sum of squares of the residuals is insensitive to the value of the parameter.

Inverse efficacy (E_{inv}) is defined as the fraction of spontaneous (ie., agonist-independent) receptor activity that can be inhibited by a given agent, as shown in the equation $E_{inv} = A_I/A_R$, where $A_I = (a_s - a_I)/a_s$ and $A_R = (a_s - a_0)/a_s$. A_R represents the relative signal that is attributable to the receptor alone, in other words, the difference between the signal observed in the presence of receptor but in the absence of agonist or antagonist (a_s) and that observed in the absence of receptor (a_0), divided by a_s. A_I represents the relative decrease induced by maximal concentrations of a given agent. This is the difference between a_s and the signal observed at a saturating concentration of ligand (a_I), divided by a_s.

In the present study, a_s and a_I represent cAMP production in the absence of AR ligand and in the presence of a maximally effective concentration of the latter, respectively. These were estimated from simultaneous analyses, as described above, for experiments carried out in membranes; for experiments carried out in whole cells the measured values were used. The parameter a_0 represents the rate of cAMP production observed with preparations lacking β_2AR in the absence of any AR ligand. The mean values of A_R were 0.78 ± 0.02 in membranes (11 experiments) and 0.64 ± 0.17 in whole cells (four experiments), and these were taken as constants in further calculations. For both membrane and whole-cell assays, values of A_I for individual ligands were averaged, and each value of E_{inv} was calculated by dividing the mean value of A_I by the mean value of A_R.

Effect of human βAR expression on the production of cAMP.

In washed membranes prepared from Sf9 cells, the level of cAMP produced in the presence of 53μM GTP was found to be greater than with cells infected for 48 hr with baculovirus encoding the human β_2AR than with either cells infected with wild-type baculovirus or uninfected cells (Fig.1). To establish that the increase in cAMP was not due to stimulation of the receptor by contaminating catecholamines that may have been present in the complete medium, experiments were carried out on cells maintained in serum-free medium for the final 18 hr and treated with alprenolol (1μM) for the final 30 min of the infection. As shown in Figure 1, *inset*, the receptor-mediated increase was still observed under these conditions. An increase was also observed with serum-deprived cells that had not been treated with alprenolol. It follows that the β_2AR is spontaneously active in washed membranes. In addition to the receptor-mediated, agonist-independent increase in adenylyl cyclase activity observed in membranes from cells expressing β_2AR, the βAR agonist isopreterenol further stimulated the enzyme. The agonist was without effect on membranes from cells that did not express the receptor (Figure 1).

Infections for 48 hr at various multiplicities of infection yielded levels of β_2AR expression ranging from 0.05 to 45 pmol/mg protein. As shown in Figure 2, the agonist-independent and the net isoproterenol-stimulated and forskolin-stimulated increases in cAMP production all were linearly correlated with the level of β_2AR expression ($r^2 = 0.97$, $r^2 = 0.99$, and $r^2 = 0.98$ respectively).

Inhibition of receptor-stimulated cAMP production.

Alprenolol partly reversed the increase in cAMP production associated with the expression of the β_2AR, whereas isoproterenol stimulated adenylyl cyclase activity > 2-fold in the same preparations (Figure 3). When membranes containing β_2AR were treated with both isoproterenol and alprenolol, a net decrease was observed in the level of enzymic activity, similar to that found with alprenolol alone. Membranes from cells lacking the receptor exhibited similar levels of enzymic activity in the presence and absence of alprenolol ($p=0.82$), indicating that the inhibition was due to an interaction between alprenolol and the receptor. To ensure that competition with serum catecholamines in the culture medium was not responsible for the observed inhibition, experiments were carried out on cells maintained in serum-free medium. Alprenolol was found to inhibit adenylyl cyclase in a dose-dependent manner in washed membranes from cells cultured under such conditions (Figure 4). This inhibition was observed even when cells were treated with alprenolol for 30 min. before harvesting. Similarly, treatment of cells maintained in complete medium with 1 μM alprenolol for 30 min before harvesting of membranes did not prevent the decrease in cAMP production with alprenolol, indicating that the inhibition observed under normal experimental conditions was not dependent upon contaminants in the medium.

Propanolol also was found to inhibit the β_2AR-associated increase in adenylyl cyclase activity (Figure 5). The magnitude of the response appeared to be limited by the receptor-induced increase in cAMP. In absolute terms, the inhibition of the enzyme by propranolol (Figure 5) or alprenolol increased with the receptor-dependent increased in cAMP. At the lowest concentrations of receptor investigated (12-hr infection, 0.03 ± 0.02 pmol of β_2AR/mg of protein), the inhibition could not be observed reliably, although a small amount of stimulation was

observed under such conditions in r sponse t isopr terenol. The maximal l v ls f both th stimul tion and the inhibition of adenylyl cyclas thus app ar to be dependent upon the density of β_2AR in the membrane.

A variety of AR ligands were characterized with respect to agonist-independent inhibitory potency, binding affinity, and inverse efficacy (Figure 6 and Table 1). The production of cAMP in the membranes from cells expressing β_2AR was increased 4.5-fold on average, relative to that in membranes from cells lacking the receptor; none of the agents tested decreased cAMP production to a level below that observed in membranes from cells lacking. The βAR ligands tested varied considerably with respect to their effects on adenylyl cyclase activity, as illustrated in Figure 6. Timolol caused the greatest inhibition and dichloroisoproterenol the least. Isoproterenol typically stimulated enzymic activity 1.5 to 2.5-fold.

The inhibitory effects of nine different AR ligands on the binding of ^{125}I-CYP and on the production of cAMP in membranes from β_2AR-expressing Sf9 cells are summarized in Table 1. In general, the rank orders of affinity (K_d) and potency (EC_{50}) are in agreement with each other. For each inhibitory βAR ligand tested, the EC_{50} value exceeded the K_d value (Table 1).

There was no obvious relationship between the inverse efficacy of a given ligand and its EC_{50}, K_d, or EC_{50}/K_d value. If the observed inhibition were due to competition between the ligands tested and contaminating catecholamines, a correlation between E_{inv} and K_d would be expected. Timolol, propranolol, and alprenolol all yielded similar values with respect to potency and binding affinity, but the inverse efficacy of alprenolol was significantly less than that of either timolol ($p = 0.002$) or propranolol ($p = 0.032$). Pindolol was less potent than any of those three agents but

xhibit d an invers fficacy similar to that of alpr nolol. Of the ßAR ligands tested, dichl r isopr t renol was th weak st with r spect t potency, affinity, and inverse efficacy.

Three αAR antagonists, ie., phentolamine, prazosin, and yohimbine, were also tested. Although they bound with affinities several orders of magnitude weaker than those of most of the ßAr antagonists tested, these agents inhibited $ß_2$AR-induced cAMP production by at least 50%, further demonstrating the lack of correlation between E_{inv} and K_d. For example, the inverse efficacy of yohimbine was greater than that of labetalol, even though the K_d of the former was approximately 4 orders of magnitude higher than that of the latter. Precise values for EC_{50} and E_{inv} could not be reliably determined for the αAR antagonists, because enzymic activity was still decreasing at the highest concentrations of those ligands tested (≥100 μM) and y_{∞} was therefore undefined. Although an analogous problem was encountered in the binding experiments, values of K_d could be estimated by assuming that the nonspecific binding of ^{125}I-CYP was the same with both ßAR and αAR ligands. Comparison of values of K_d with the incomplete dose-response curves obtained with prazosin, phentolamine, and yohimbine suggests that the rank orders of affinity and potency are the same for αAR antagonists, consistent with the effects of ßAR ligands.

Competitive effects among ligands with different inverse efficacies.

The agonist-independent inhibition of adenylyl cyclase activity by timolol could be partly blocked by less efficacious agents. At 10 nM timolol, the inhibition of cAMP production was nearly maximal (see Figure 6), but the additional inclusion of labetalol (100 μM) partially

revers d that inhibition (Figur 7A). Similarly, high r concentrations f timolol wer required to inhibit cAMP production in th pr s nce f dichloroisoproterenol at 100 μM (Figure 7B). It is clear that dichloroisopr t renol was not acting as a partial agonist, because it reduced the increase in enzymic activity attributable to the spontaneous activity of the receptor. The inhibition of the effect of timolol by ligands with relatively low inverse efficacies suggests that the different antagonists compete for a common binding site and that the incomplete effects of ligands such as labetalol do not arise from a failure to saturate the receptor. The latter point is supported by the observation that both labetalol and dichloroisoproterenol fully inhibit the specific binding of ^{125}I-CYP (Table 1).

Example 2: 5HT-2C Receptors

Experimental Procedures

Construction of Recombinant Baculovirus

The recombinant baculovirus used for expression of the rat 5HT-2C receptor was provided under license from Texas A & M University System. The virus was constructed using a synthetic DNA fragment encoding the rat 5HT-2C receptor, based on the published sequence of the cloned cDNA from choroid plexus (Julius, D., et al., 1988, *Science*, 241:558-564). The synthetic cDNA was prepared and its sequence verified by Allelix Biopharmaceuticals (Mississauga, ON). The cDNA was inserted into the IpDC-126 baculovirus transfer vector and a recombinant baculovirus was produced and purified as previously described (O'Reilly, D.R., 1992, *supra*). Stocks of baculovirus containing the 5HT-2C receptor coding sequence were amplified and used to express 5HT-2C receptors in Sf9 cells.

Cell culture and infecti n with recombinant baculovirus.

Buffer chemicals and pr tease inhibit rs were purchased from Sigma and cell culture media from Gibco. Unlabelled ligands were supplied by Research Biochemicals International (Natick, USA), with the exception of RU4969 which was a gift from Roussel Uclaf. The [3H]mesulergine (78-82 Ci/mM) was purchased from Amersham and [3H]myo-inositol (10-20 Ci/mM) were purchased from NEN Dupont. The ion exchange resin AX-18 was supplied by Bio-Rad.

Cell culture and membrane preparation.

Sf9 cells were cultured in 50 ml batches in 250 ml shaker flask (27 °C in Sf-900 II serum free medium/50 mg/ml of gentamicin sulfate). Cells were grown to a cellular density of $3x10^6$ cells/ml and infected with the 5HT-2C encoding baculovirus or with wild type AcMNPV virus at a multiplicity of infection of 2. Viral stocks were in Grace's insect medium containing 10% fetal bovine serum (Hyclone). For analysis of ligand binding to membrane preparations, cultures were harvested at 48 hr post-infection, lysed and a post-nuclear membrane pellet prepared as described (Labrecque, J., et al., 1992, *FEBS Lett*, 304:157-162) and stored at -80 °C. Protein concentrations were determined by nitrocellulose amido black method as described by Schaffner (1973, *Analytic. Biochem.*, 56:502-514). For measurements of radio-ligand binding to intact cells and of inositol phosphate production, cultures were processed at specified times post-infection as described below.

Radio-ligand binding assays.

Membranes for radio-ligand binding assays were thawed on ice and resuspended in binding buffer (5mM Tris/HCl, pH 7.4, 15mM $MgCl_2$, 2 mM EDTA, 0.1% ascorbic acid/protease inhibitor; 5 mg/ml leupeptine, 10 µg/ml aprotinin, 20 mg/ml benzamidine, 50 mg/ml TPCK and 50 mg/ul trypsin inhibitor) by homogenization in a Potter homogenizer. The membranes (5 mg) were incubated with [^{3}H]mesulergine for 1 hr at 27 °C in a volume of 540 µl, and reactions terminated by vacuum filtration over GF/C filters and washing. Non-specific binding was determined in the presence of 20 mM mianserine or metergoline. Bound radioactivity was measured on filters impregnated with melt on scintillation using a

Wallac beta-scintillation counter (Wallac-Oy, Turku, Finland). Saturation binding assays employed increasing concentrations of [^{3}H]mesulergin, while competition assays used increasing concentrations of unlabelled drugs with 3nM [^{3}H]mesulergine.

The measurement of total receptor number in time course experiments was carried out with intact cells harvested at specified times post-infection. Briefly, cells were washed with PBS, resuspended PBS or binding buffer and incubated (10,000 cells per assay) in 540 ml with a saturating concentration (20 nM) of [3H]mesulergine. Total and non-specific binding were measured as described for membrane binding assays.

Inositol phosphate production

Growing cell cultures were pre-labelled for 24 hr with 1 mCi/ml [^{3}H]myo-inositol prior to infection, and the labelled cells were then transferred to 50 ml shaker flasks for experiments. Cell suspension assays in replicate (2 or 3 as specified) were started by addition of labelled cells to multi-well plates containing drugs to be tested, followed by immediate mixing. The cells were then incubated for 20 min. at 27 °C in a shaking incubator, and the incubations stopped by addition of perchloric acid. Total [^{3}H]-labelled inositol phosphates (IP) were measured by scintillation counting following isolation by anion exchange chromatography on AX-18 resin, as described (Fargin, A., et al., 1989, *J. Biol. Chem.*, 264:14818-14852) and radioactivity measured by scintillation.

Data analysis.

Saturation and competition binding data were analyzed by non-linear regression using the LIGAND™ computer program (Munson, P.J., and Rodbard, D., 1980, *Analytic Biochem.*,

107:220-239). Dose response curves for IP production were analyzed using a four-parameter non-linear sigmoid curve regression (InPlot™ 4.0) with a fixed "Hill" slope of 1 for analysis of agonist and -1 for inverse agonist analysis. *F test* were used in the program to estimate best fit (with floating or fixed parameters). EC_{50} values were extrapolated with the standard error estimate (SE) from best fit estimate (Table 2). Efficacy (agonists) and inverse efficacy (antagonists) for each drug were compared statistically by a two tailed *t test* (heteroscedastic, a = .05).

Expression of functional rat 5HT-2C receptors in Sf9 cells.

A recombinant baculovirus containing the synthetic 5HT-2C receptor DNA was used to express the receptor in cultures of Sf9 insect cells. Receptor levels, as measured in membrane preparations by binding of [^{3}H]metergoline, increased over the course of the infection to peak at 48 hr post-infection. Membranes prepared at this time contained 27 pmol/mg protein of metergoline binding sites (KD = 2nM), and bound serotonergic ligands with appropriate affinities (see Table 2). Analysis of the binding data indicated a single class of sites for all of the ligands except 5HT and DOI, which displayed both high and low affinity sites.

The ability of the expressed receptor to modulate endogenous phospholipase C activity in Sf9 cells was assessed at various times post-infection by measuring the production of total IP in intact cells pre-labelled for 24 hr with [^{3}H]myo-inositol. As shown in Figure 8, the ability of the agonist 5HT to stimulate IP production varied over the course of the infection. The agonist had no significant effect at 12 hr or 72 hr post-infection, nor in cells infected with wild-type baculovirus. At 20 hr and 38 hr post-infection with the recombinant virus, 5HT

stimulated the synthesis of total IP, the extent of stimulation being greatest at 20 hr (30% over basal versus 13% at 38 hr). The effect of 5HT was blocked by the antagonist mianserine (Figure 8), but was unaffected when cells were pretreated with pertussis toxin. These results indicate that the expressed 5HT-2C receptor couples to Gq-like G protein(s) in Sf9 cells to modulate endogenous phospholipase C activity.

Spontaneous activity of the expressed 5HT-2C receptor.

The results in Figure 8 show that the basal level of IP production, as measured in the absence of added agonist, increased between 12 hr and 38 hr post-infection, and at 38 hr was over four times higher in cells expressing the 5HT-2C receptor than in wild type-infected controls. This increase in basal activity paralleled a rise in receptor levels over the time course of the infection, as measured by [^{3}H]mesulergine binding to intact cells (inset Figure 8). Basal IP production at 72 hr fell to a level similar to that in cells infected with wild-type virus, despite the high level of receptors. The cell viability of 70% observed at 38 hr gradually dropped to about 10% of viable cells at 72 hr, as measured with 0.4% Trypan blue. The drop in activity presumably reflects a generalized disruption of cell function which occurs late in the viral infection in association with cell lysis.

The increased basal activity apparent in Figure 8 would not appear to be due to the presence of 5HT in the medium, since these experiments were carried out in serum-free medium lacking 5HT, and the cells were washed extensively before measurement of IP production. The findings are consistent with the view that the expressed 5HT-2C receptor exhibits spontaneous, agonist-independent activity in Sf9 cells, and that this activity is amplified as receptor levels increase.

Modulation of 5HT-2C rec ptor activity by drugs. Th results in Fig. 8 show that at 20 hr and 38 hr th antagonist mianserin reduc d 5HT-stimulat d IP synthesis to a level below that measured in the absence of agonist; furthermore, basal activity was decreased by the antagonist alone, suggesting that mianserine inhibits spontaneous activity of the 5HT-2C receptor in Sf9 cells. The extent of inhibition by 10 mM mianserine was greater at 38 hr (roughly 30%) than at 20 hr (10%), the reverse order of that observed for 5HT stimulation of IP synthesis. These results defined optimal times post-infection to analyze the effects of agonists (20 hr) and antagonists (38 hr) on the coupling of the 5HT-2C receptor to IP production.

The results in Figure 9 show dose-response curves for agonist-stimulation of IP production measured at 20 hr post- infection. 5HT and the DOI stimulated IP synthesis to similar degrees (Emax = ca. 30%), while RU24969 acted as a partial agonist on this response (Emax = 12%). The EC_{50}s for the three agonists were consistent with their affinities as measured in competition binding studies (Table 2), with the rank order of potency of 5HT > DOI >> RU24969.

The effects of a series of serotonergic antagonists on basal (ie. spontaneous) 5HT-2C receptor activity were analyzed at 38 hr post-infection; the results are shown Figure 10 (A and B) and summarized in Table 2. All of the antagonists tested, with the exception of spiroxatrine, produced a dose-dependent inhibition of basal IP production. The EC_{50} values for this inhibition were in good agreement with Ki values measured in competition binding, and showed the same rank order (metergoline > methysergide > ritanserine | mianserine = mesulergine > ketanserine > spiperone) (Table 2).

The magnitude of the inhibition of basal activity (Emax) was dependent on the antagonist, with maximal inhibition (32%) being observed with mianserin (Figure 10 and Table 2). The rank order of inverse efficacy (Emax) was mianserine > metergoline = ritanserine > methysergide = spiperone = mesulergine > ketanserine. This is clearly different from the rank order observed for potency and binding affinity for this series of antagonists.

Spiroxatrin was th only ligand tested which had n significant effect on IP production in th s experiments. In contrast, spiroxatrine was able to inhibit both [^{3}H]mesulergine binding to the expressed 5HT-2C receptor (Table 2) and 5HT- stimulated IP production (Figure 11, top panel) with comparable potencies, consistent with competitive antagonist activity. The action of spiroxatrine was further characterized by testing its ability to modify the effect of mianserine on basal 5HT-2C receptor activity at 38 hr post-infection. As shown in Figure 11 (bottom panel), 10 mM spiroxatrine caused a rightward shift in the dose-response curve for the inhibition of basal activity by mianserine. These findings illustrate that spiroxatrine behaves as a competitive antagonist for both 5HT-induced coupling and for mianserine-induced inhibition of spontaneous activity for the 5HT-2C receptor in Sf9 cells, but has no intrinsic efficacy either as an agonist or as an inverse agonist.

2135253

Inhibition of β_2AR-associated increase in membrane adenylyl cyclase activity by AR ligands

Sf9 cells were infected for 48 hr with baculovirus encoding the β_2AR, to yield 23 ± 2 pmol of receptor/mg of membrane protein.

βAR ligands	EC_{50}[a]	n[b]	K_d[a]	n	E_{max}[c]	n
	nM		nM			
Alprenolol	6.7 ± 1.1	6	0.32 ± 0.06	3	0.68 ± 0.02	6
Propranolol	7.9 ± 1.2	4	0.36 ± 0.08	3	0.82 ± 0.04	4
Timolol	10.0 ± 1.8	6	0.29 ± 0.07	2	0.91 ± 0.04	6
Pindolol	36 ± 8	6	0.71 ± 0.01	3	0.65 ± 0.04	6
Labetalol	61 ± 44	4	10.6 ± 2.8	2	0.40 ± 0.03	4
Dichloroisoproterenol	760 ± 396	4	220 ± 92	1	0.09 ± 0.11	4
αAR ligands			K_d[d]	n	E_{max}	n
			μM			
Prazosin	e		2.3 ± 1.1	2	≥0.7	3
Phentolamine	e		31 ± 16	2	≥0.5	3
Yohimbine	e		592 ± 83	2	≥0.5	3

[a] Errors accompanying EC_{50} and K_d values reflect the range over which the sum of squares is insensitive to the value of the parameter.

[b] n, number of experiments.

[c] Values are mean ± standard error.

[d] Specific binding was not completely inhibited at the highest concentrations of these ligands used (2.0 mM) and nonspecific binding was set equal to that estimated from concurrent experiments with βAR antagonists.

[e] Maximal effect was not defined at the highest concentrations tested (0.1–1.0 mM), and therefore EC_{50} could not be estimated reliably.

47-A

TABLE 1

Pharmacology of 5HT-2C receptor expressed in Sf9 cells.

Results are expressed as mean ± SE for at least 3 experiments and E_{max} represent the % of IPs production over the unstimulated basal level. Non specific binding was determined with 100 µM metergoline for mianserine competition binding or with 100 µM mianserine for all other drugs. Maximal efficacy and maximal inverse efficacy (Emax) for each drug were compared statistically by a two tailed t test (heterocedastic, $\alpha = 0.05$). Failure of t test was considered as an equivalence of E_{max} between drugs. Agonist activity on IPs production was measured at 20 h where as antagonist activity was measured at 38 h post-infection.

	Binding parameters	IPs responses	
Antagonists	**‡ pKi**	**‡ pEC50**	**E_{max}**
mianserine	8.63 ± 0.05	7.45 ± 0.24	-32.2 ± 2.6
metergoline	9.22 ± 0.10	8.81 ± 0.20	-26.3 ± 3.5
ritanserine	8.89 ± 0.10	7.75 ± 0.36	-23.4 ± 4.3
methysergide	9.12 ± 0.05	7.95 ± 0.36	-21.1 ± 4.0
spiperone	5.92 ± 0.10	5.08 ± 0.41	-20.0 ± 2.7
mesulergine	# 8.70 ± 0.05	7.35 ± 0.46	-17.6 ± 3.6
ketanserine	7.69 ± 0.02	6.70 ± 0.21	-16.0 ± 3.6
spiroxatrine*	5.17 ± 0.08	5.04 ± 0.99	2.0 ± 2.0
Agonists	**pKi**	**pEC50**	**E_{max}**
RU24969	6.00 ± 0.20	5.89 ± 0.41	12.3 ± 1.5
5HT	7.23 ± 0.30 (high)	7.77 ± 0.71	31.6 ± 4.3
	6.23 ± 0.30 (low)		
DOI	7.33 ± 0.10 (high)	7.00 ± 0.21	33.3 ± 4.1
	6.15 ± 0.10 (low)		

‡ EC_{50} and Ki values are presented as: (-) log (Ki or EC_{50}) = pKi or pEC_{50}.
* Spiroxatrine does not induce an effect on PI turnover, but compete with 5HT activation, pIC_{50} = 5.04.
# Mesulergine KD value from saturation.

TABLE 2

47-B

Th embodiments of th inv ntion in which an exclusiv property r privil ge is claimed ar defined as follows:

1. A method of testing chemical compounds for their abilities to inhibit chemical-messenger-independent activity of G protein-coupled receptors involving:

(a) expressing DNA encoding a G protein-coupled receptor in a cell expression system in such a manner as to generate a reproducible "window of chemical-messenger-independent activity" that allows for discrimination of chemical compounds based on relative ability to inhibit chemical-messenger-independent activity of said G protein-coupled receptor;

(b) measuring a quantifiable parameter using biochemical or other assay procedures that indicate the agonist-independent activity of said receptor in said system comprising whole cells or membrane fragments containing G protein, an appropriate effector, and cloned G protein-linked receptor;

(c) contacting a test-compound with the system under conditions permitting interaction of the test-compound with said receptor; and

(d) measuring the change, if any, of the quantifiable parameter which reflects the ability of the test compound to inhibit the chemical-messenger-independent activity of the G protein-coupled receptor.

2. The method according to claim 1, wherein said G protein-coupled receptor gene is encoded by a cDNA.

3. A method according to claim 1, wherein whole cells or membrane fragments thereof, are derived from a baculovirus expression system in Sf9 cells.

4. A method according to claim 3, wherein said receptor is a β-adrenergic receptor.

5. A method according to claim 1, wherein said receptor is a serotonin receptor.

6. A method according to claim 1, wherein said receptor is a β-adrenergic receptor.

7. A method according to claim 1, wherein said receptor is a serotonin receptor.

8. A test kit including:

(a) whole cells or membrane fragments thereof, comprising cloned G protein-coupled receptors, functionally expressed at a concentration demonstrating a "window of chemical-messenger-independent activity".

9. The test kit according to claim 8, wherein whole cells or membrane fragments thereof, are derived from a baculovirus expression system in Sf9 cells.

10. The test kit according to claim 9, wherein said G protein-coupled receptor is a β-adrenergic receptor.

11. The test kit according to claim 9, wherein said G protein-coupled receptor is a serotonin receptor.

12. A test kit utilizing the method as claimed in claim 1.

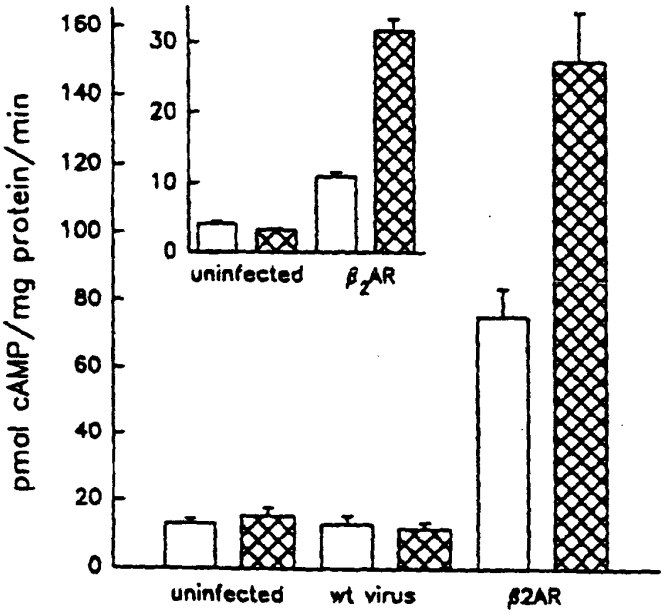

pmol cAMP/mg protein/min
160
140
120
100
80
60
40
20
0
30
20
10
0
uninfected
β_2AR
uninfected
wt virus
β2AR

FIGURE 1

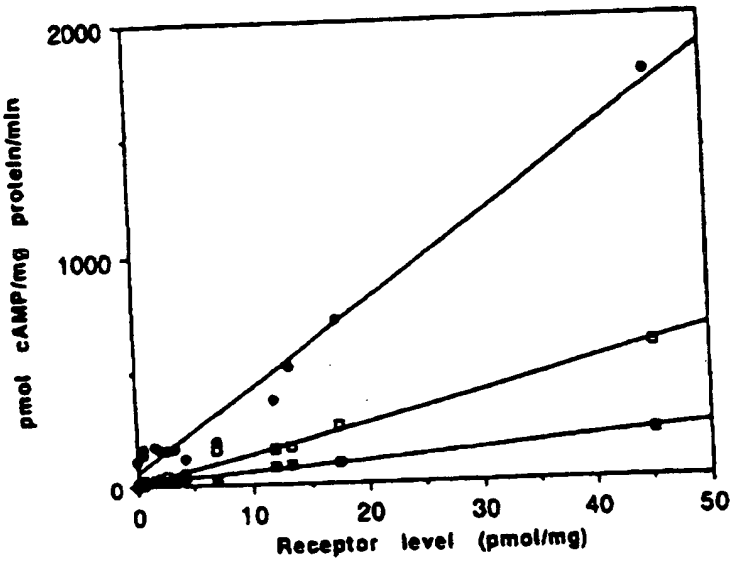

2000
1000
0
pmol cAMP/mg protein/min
8
0
10
20
30
40
50
Receptor level (pmol/mg)

FIGURE 2

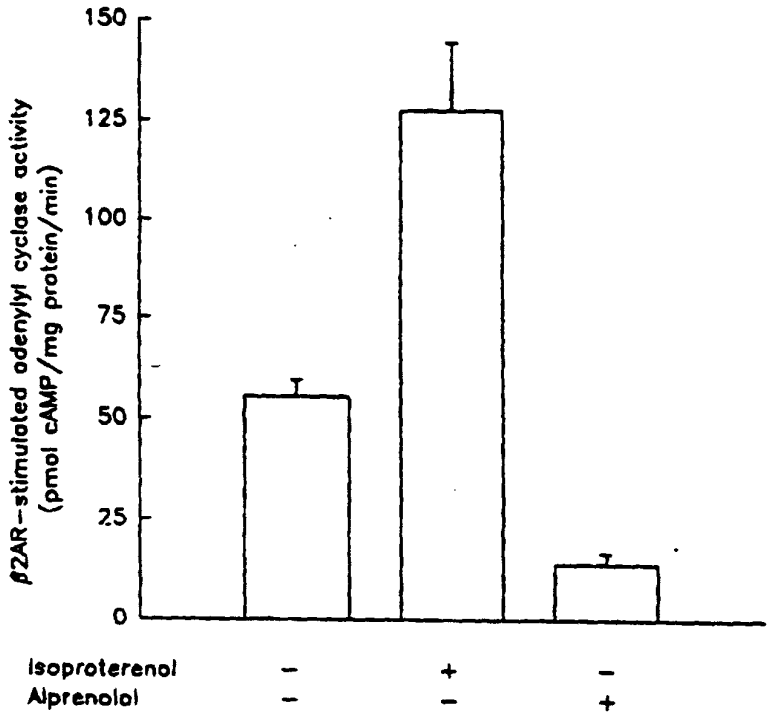

150
125
100
75
50
25
0
β2AR-stimulated adenylyl cyclase activity
(pmol cAMP/mg protein/min)
Isoproterenol
Alprenolol
–
–
+
–
–
+

FIGURE 3

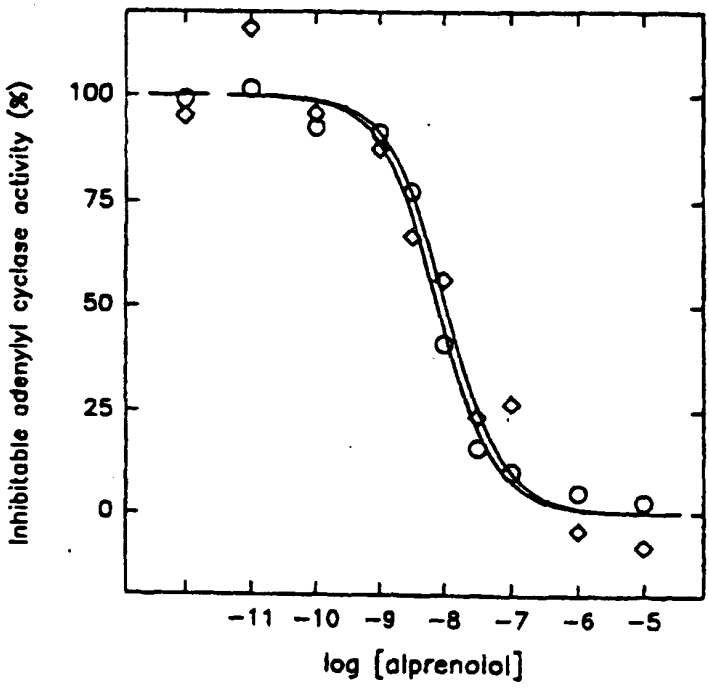

Inhibitable adenylyl cyclase activity (%)
100
75
50
25
0
−11 −10 −9 −8 −7 −6 −5
log [alprenolol]

FIGURE 4

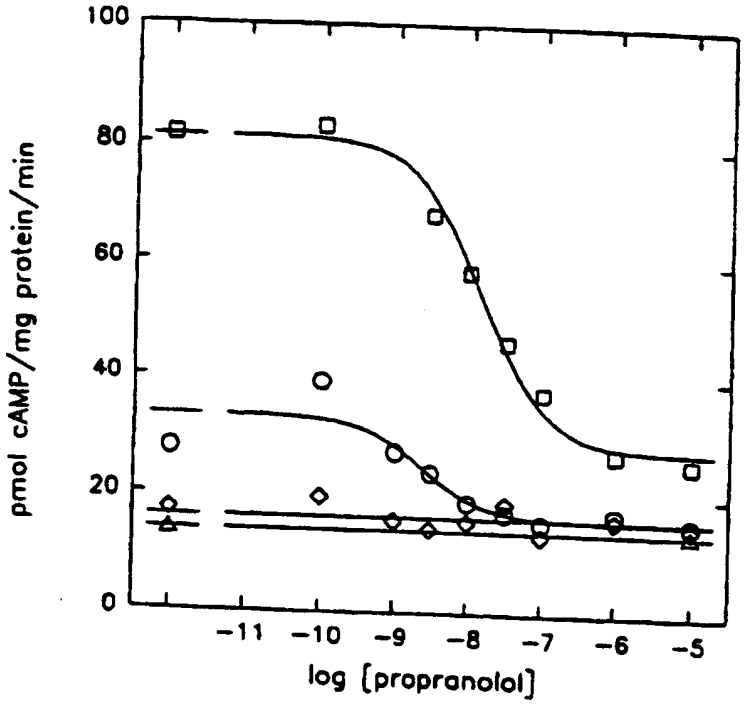

100
80
60
40
20
0
pmol cAMP/mg protein/min
−11
−10
−9
−8
−7
−6
−5
log [propranolol]

FIGURE 5

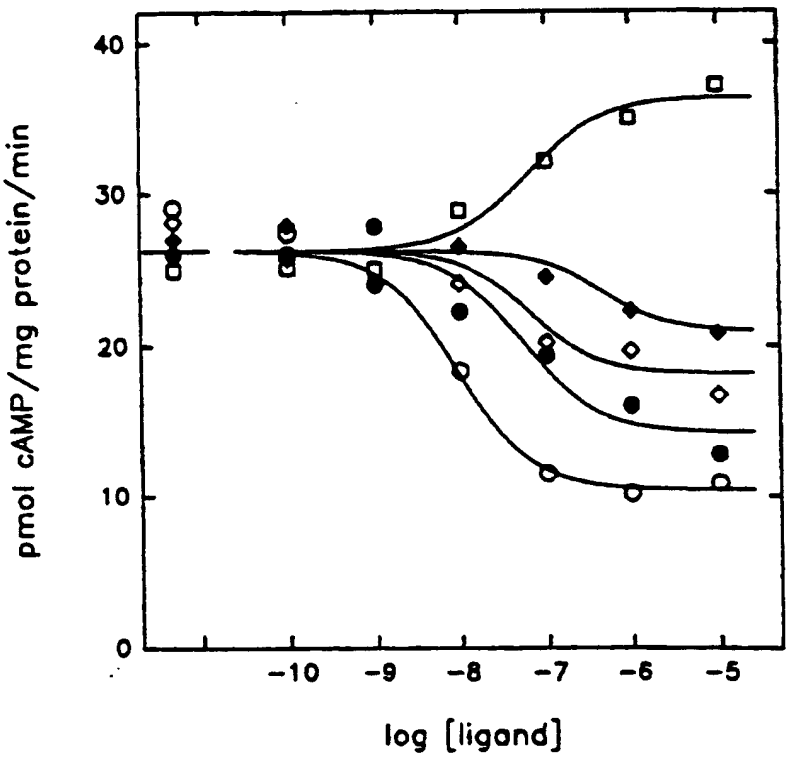


FIGURE 6

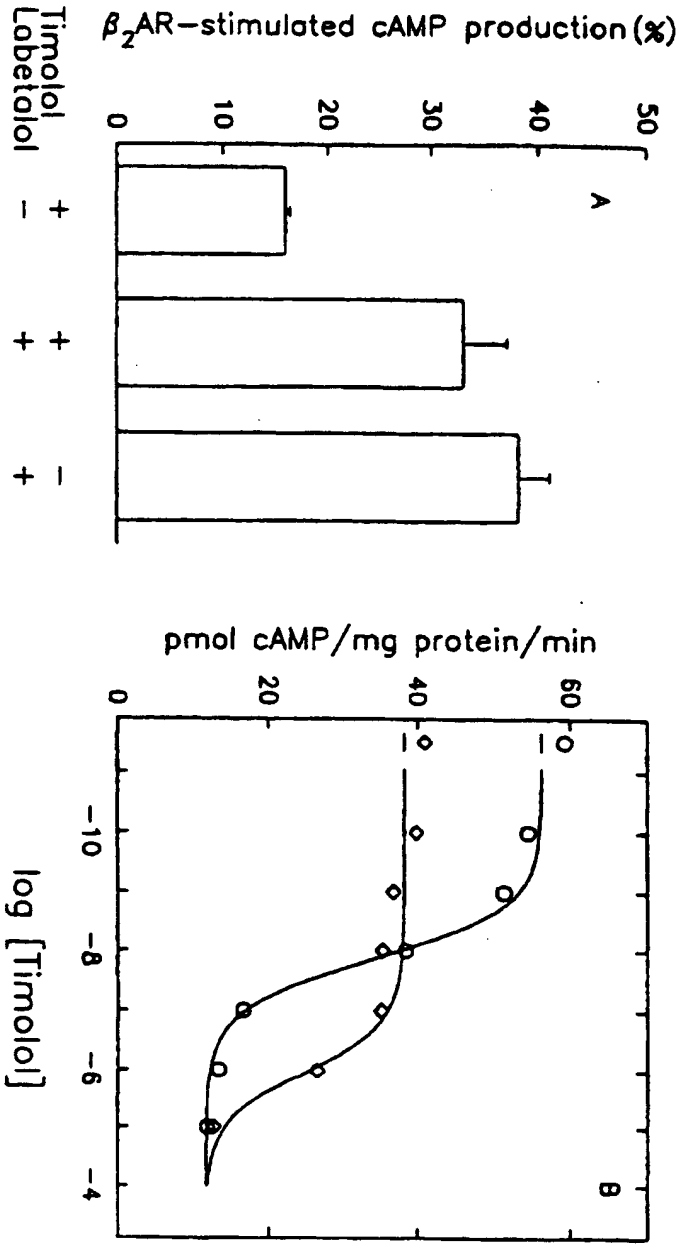
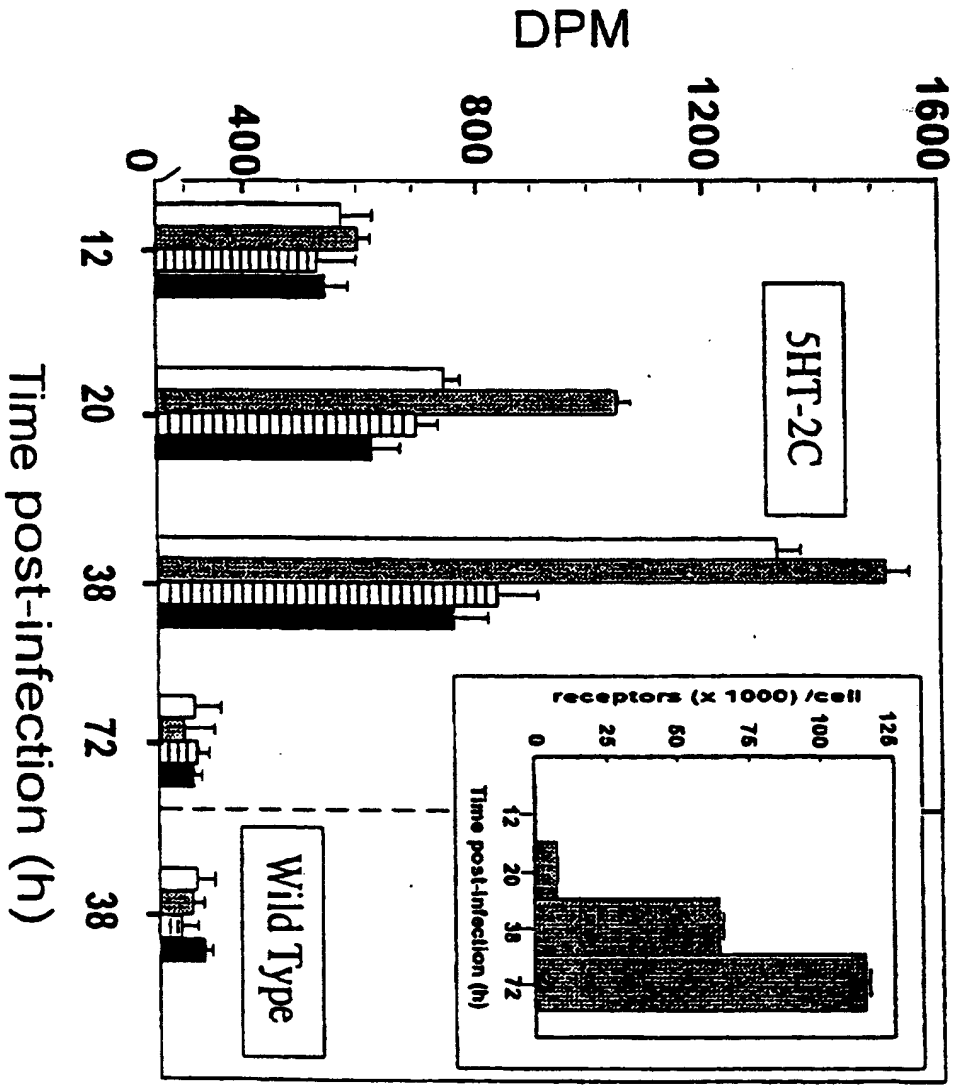

β_2AR–stimulated cAMP production (%)
Timolol
Labetalol
0
10
20
30
40
50
A
– +
+ +
+ –
pmol cAMP/mg protein/min
0
20
40
60
log [Timolol]
–10
–8
–6
–4
B

FIGURE 7


DPM
0
400
800
1200
1600
5HT-2C
Wild Type
Time post-infection (h)
12
20
38
72
38
receptors (x 1000) /cell
0
25
50
75
100
125
Time post-infection (h)
12
20
38
72

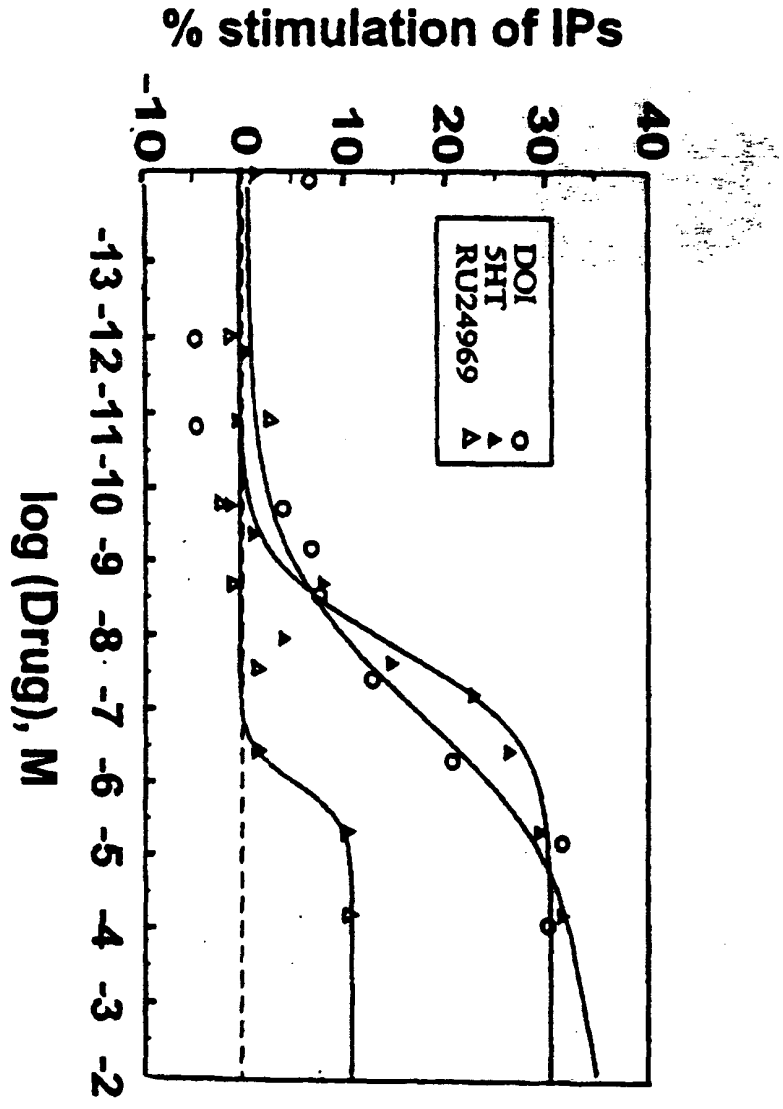

% stimulation of IPs
-10
0
10
20
30
40
DOI
5HT
RU24969
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2
log (Drug), M

FIGURE 9

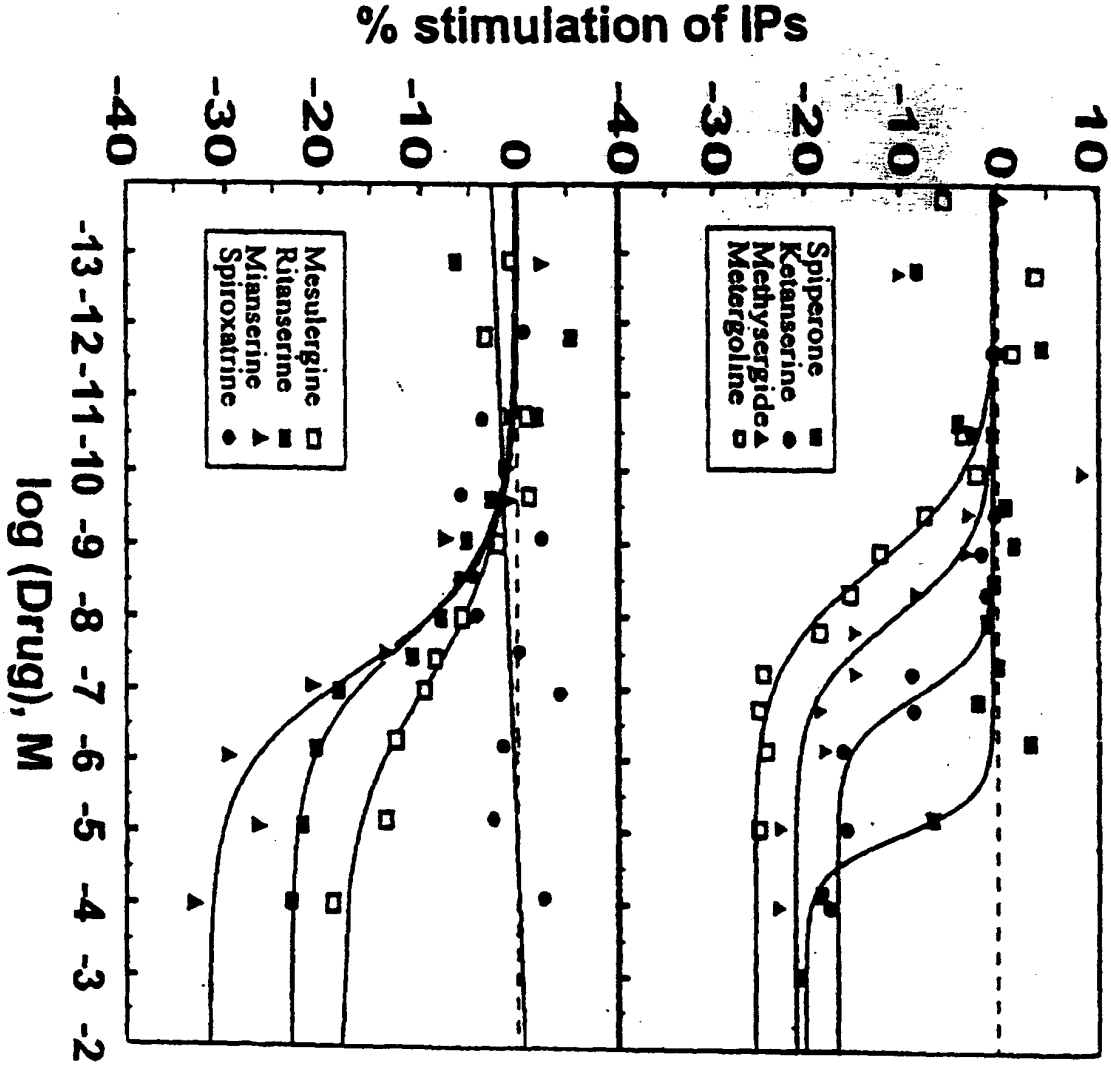

% stimulation of IPs
10
0
-10
-20
-30
-40
Spiperone
Ketanserine
Methysergide
Metergoline
Mesulergine
Ritanserine
Mianserine
Spiroxatrine
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2
log (Drug), M

FIGURE 10

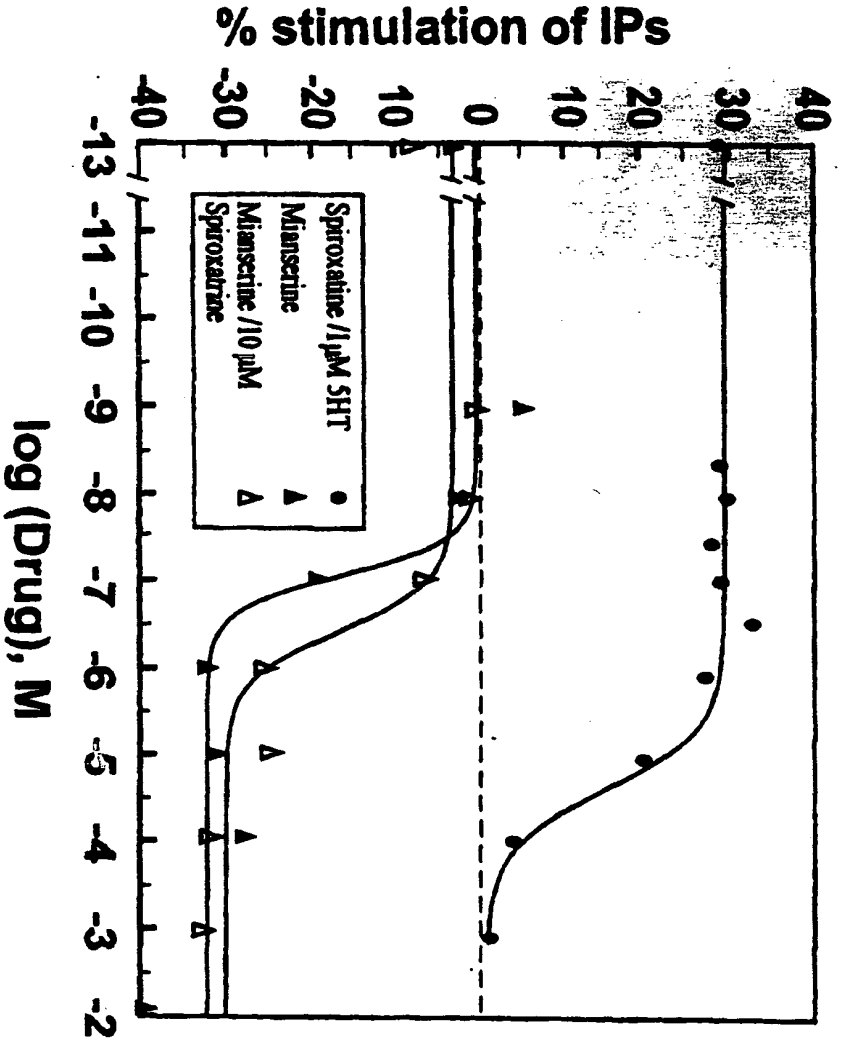

% stimulation of IPs
40
30
20
10
0
-10
-20
-30
-40
-13 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2
log (Drug), M
Spiroxatine /1μM 5HT
Mianserine
Mianserine /10 μM
Spiroxatrine

FIGURE 11